



# Effects of Long-Term Lead Exposure on Antioxidant Enzyme Defense System in Organs of Brown Hare (*Lepus europaeus* Pallas) as a Bioindicator of Environmental Pollution in Croatia

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## Abstract

In Croatia, Podravina is a well-known lead-polluted region due to the intensive exploitation of natural gas, a highly developed agricultural industry, and a traffic hub with several heavily traveled roads. It represents a natural environment with a great variety of wildlife, especially hares (*Lepus europaeus* Pallas), which may serve as an indicator for environmental quality assessment. This study was conducted to estimate the bioaccumulation of lead in hare liver, kidney, muscle, and brain during long-term exposure and its impact on the oxidative status of the organism and to investigate a possible lead exchange ionic mechanism in the brain. In the organs of two hare groups (experimental from polluted area and control from the island of Krk), Ca, Fe, Mg, Na, lead concentrations, and antioxidant enzyme defense system were analyzed. The accumulation of lead was highest in the brain (3.7-fold higher compared to the control group) and lowest in the liver (1.6-fold higher compared to the control group). Kendall-Tau and multiple regression analysis showed that the increased lead content caused a stronger exchange of Ca and Na ions in the brain. We proposed that lead either mobilizes intracellular cation stores or causes competitive displacement of Ca from the binding site in intoxicated cells. A linear predictive model for cell intoxication by lead was proposed, where GPx and SOD were predominantly influenced by long-term lead exposure. The presented results showed that long-term lead exposure in hares negatively affected their oxidative status and caused the strongest toxicity in the brain and muscles, making their survival and/or population vulnerable.

**Keywords** Biomonitoring · Hare · Lead · Minerals · Oxidative stress enzymes

## Introduction

Environmental contamination by toxic metals poses a threat to “man and biosphere,” reducing agricultural productivity and damaging the health of the ecosystem [1]. Among them, lead (Pb) is the most common nonessential heavy metal and

a very potent environmental pollutant emerging in areas near smelters, refineries, mining areas, and heavily traveled roads. In addition, due to the faster urbanization process and industrial development at the start of the twentieth century, a growing amount of lead was released into the environment. The current situation is improved because of awareness of lead usage, but the lead levels in soil remain high in some areas across Europe [2].

Our area of interest was the Podravina region in Croatia (Molve, Kalinovac, and Stari Gradac), where natural gas and petroleum have been exploited for the last three decades. This region has the highest range of lead concentration in soil in North Croatia and produces 70% of the natural gas in Croatia [3, 4]. It is an area with a natural environment where a great variety of wildlife can be found in a relatively small area. The abundance of game such as roe deer, wild boars, hares, and wild ducks makes this hunting area interesting to both hunters and scientists. Some previous investigations confirmed that the European

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hare (*Lepus europaeus* Pallas) may serve as an indicator for environmental quality assessment based on its physiological characteristics, size, and predictiveness of human exposure [5]. European hares feed predominantly on cultivated crops, but they seem to prefer weeds and wild grasses if these are available [6]. Lead inhibits photosynthesis, water balance, and enzyme activities and lets down mineral nutrition in plants [7]. Animal tissue levels can provide very important data regarding the bioavailability of heavy metals within natural ecosystems [8]. In our previous studies, hares were investigated as a part of an ecomonitoring study to indicate the extent of environmental pollution by mercury and cadmium in Podravina [9, 10].

Lead toxicity has been reported to interfere with numerous body functions, primarily affecting the central nervous system (CNS) and hepatic, hematopoietic, and renal systems, producing possible serious disorders and potentially causing irreversible health effects [11]. Furthermore, lead is associated with neurodegenerative diseases and hypertension [12].

A common point in such diseases is the oxidative stress component that can be induced as a result of a depressed antioxidant system and/or increased reactive oxygen species. The most commonly used parameters to evaluate lead-induced oxidative damage are glutathione (GSH), antioxidative enzyme (SOD, superoxide dismutase), and glutathione-dependent enzymes (GR, glutathione reductase; GPx, glutathione peroxidase; GST, glutathione-S-transferase). They are directly involved in the neutralization of reactive oxygen species; thus, they play a crucial role as a major defense system against lead-induced toxicity in living beings. Several studies have reported alterations in antioxidant enzyme activities [13, 14]. Additionally, lead cannot readily undergo valence changes in its role in the onset of lipid peroxidation (LPO) [15, 16].

Various fundamental biological processes of the body are affected by lead ionic mechanisms and the ability of lead to replace other bivalent cations, such as Ca, Fe, and Mg, and monovalent cations, such as K and Na, which leads to a consequent reduction in ion concentrations inside the endoplasmic reticulum. This ionic mechanism mostly contributes to neurological deficits, as lead becomes competent to cross the blood–brain barrier after replacing calcium ions [17]. Disruption of the antioxidant balance and damage to critical biomolecules such as lipids, proteins, and DNA can lead to neuroinflammatory events in the brain [18].

The aim of this investigation was to evaluate the environmental lead concentrations in European hares from polluted areas compared to other areas where pollution was not expected. This study was also projected to estimate bioaccumulation patterns in different organs and to see how targeted

organs coped with oxidative stress after long-term exposure to low levels of environmental lead.

## Materials and Methods

### Study Area

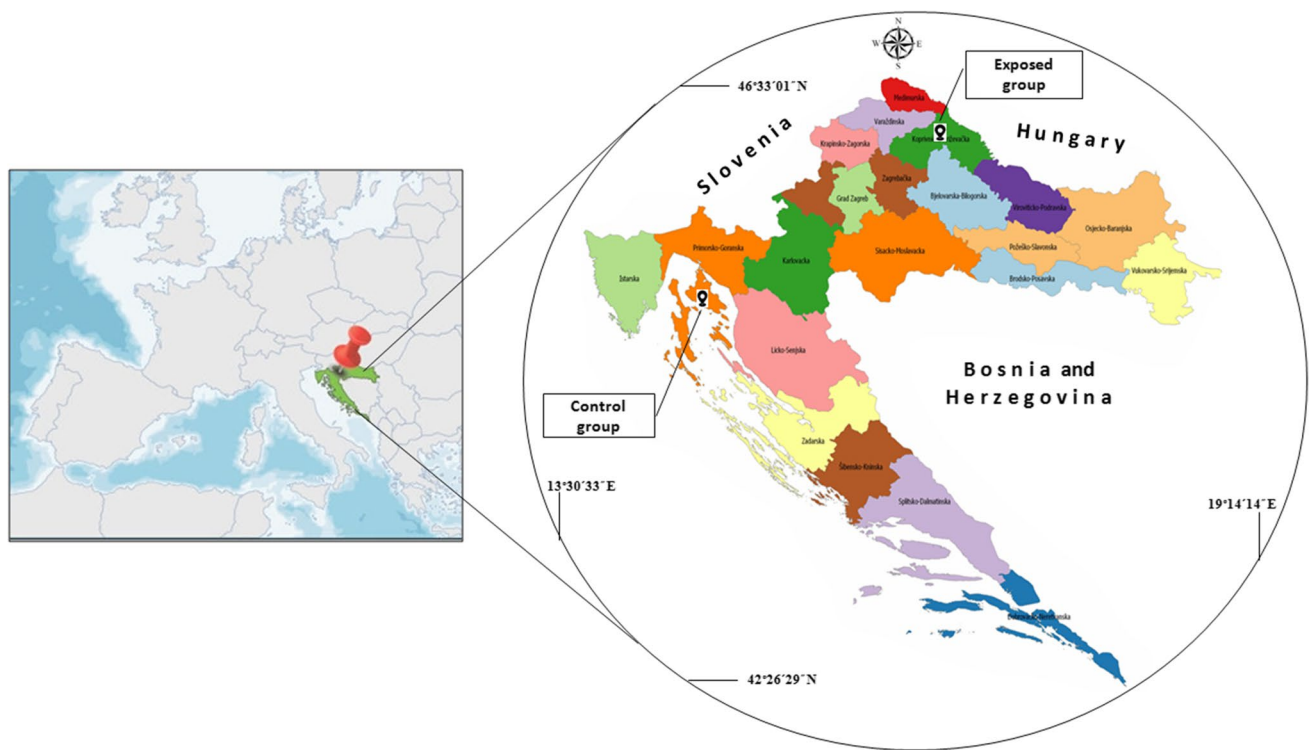
For this research, hares (*Lepus europaeus* Pallas) from two different regions in the Republic of Croatia were selected. The first study area was located in Koprivnica-Križevci County in the Podravina region of northwestern Croatia (located from 46° 21' to 46° 40' N and from 16° 39' to 17° 57' E at 96 to 190 m.a.s.l.). In addition to the intensive exploitation of natural gas, this region is also known for its highly developed agricultural industry and as a traffic hub with several heavily traveled roads. This study site has been reported with increased lead concentrations in the soil of the river Mura alluvium and remain as a consequence of lead or deposits situated more upstream where the mining activity exists [3]. For the second study area, the nonindustrial agricultural Croatian coastal region (island of Krk) was selected as a control group with no expected pollution. The island of Krk is located from 44° 56' to 45° 15' N and from 14° 26' to 14° 49' E at 75 m.a.s.l. (Fig. 1).

### Animals

The hares were divided into two groups: the experimental group from the Podravina region, where animals were exposed to an extensive industrial environment, and the control group from a coastal region (island of Krk) without agricultural and heavy industry. Five hares (in each group), 2 to 3 years of age, of both sexes (three males and two females) were selected for the experiment. The animals were shot using steel shot ammunition during the regular hunting period (October) in Croatia, in compliance with the regulations of the Croatian Hunting Law adjusted according to the European Union Directive [19, 20].

### Tissue Preparation

Immediately after the shooting, approximately 5 g of liver, kidney, muscle, and brain tissues were removed from each animal, placed in plastic bags, and sent to the laboratory in temperature-controlled chamber (4 °C). In the laboratory, sub-samples (up to 1 g wet matter) for the metal, for GSH and LPO content, and for antioxidant enzyme activity analysis were exempted from each tissue that had not been in contact with packing material. Cross-sections of each tissue were taken and washed in phosphate-buffered saline (PBS, Sigma, St. Louis, MO, USA) to remove blood remnants. In order to prevent cross-contamination during the sampling



**Fig. 1** Geographic locations of the sampling areas (experimental location, Koprivnica-Križevci County, Northwestern Croatia; control location, Croatian coastal region, the island of Krk)

procedure, glass labware sterilized at 220 °C was used as well as titanium knife and tweezers, which were cleaned in 1 M of nitric acid (Merck, Darmstadt, Germany) and rinsed in saline water for each new sample. For GSH and LPO content and enzyme analysis, tissues were weighed, frozen in liquid nitrogen, and stored at –80 °C prior to analysis, while for the metal content determination, weighed tissues were dried at 105 °C for 5 h.

### Metal Content Analysis

After drying, the tissues were digested with conc. nitric acid (Merck, Darmstadt, Germany) and 30% hydrogen peroxide (Medic, Zagreb, Croatia) and wet combusted in a microwave oven (MLS-1200 Mega Microwave Digestion System, Milestone, Sorisole, Italy) under the following conditions: 5 min, 300 W; 0.5 min, 0 W; 5 min, 600 W; and 1 min, ventilation. After cooling, the homogenates were transferred to 50-mL flasks and diluted with ultrapure water (Siemens Water Technologies Corp, Warrendale, PA, USA).

The Pb concentration was determined using the graphite furnace atomic absorption spectrometer, while the concentrations of Ca (422.7 nm), Fe (248.3 nm), Mg (285.2 nm), and Na (589 nm) were determined using the flame atomic absorption spectrometer AAS800 (Perkin Elmer, Waltham, MA USA). The quantification was done using the external

standard method, with standard solutions prepared in the same acid matrix. Analytical blanks were prepared and run in the same way as the samples. Linearity of calibration curves (five concentration levels for each element) was achieved in the concentration range 1–10 mg/L for Ca, 2–10 mg/L for Fe, 0.5–2 mg/L for Mg, 0.5–3 mg/L for Na, and 5–50 µg/L for Pb with a regression coefficient of  $R^2 > 0.995$ . Limit of detection (LOD) and limit of quantification (LOQ) for each metal were calculated according to the equation  $LOD = 3.3 r/S$  and  $LOQ = 10 r/S$ , where  $r$  is the standard deviation of the blank (calculated over ten injections of the blank) and  $S$  is the slope of the calibration curve. The quantification limits were 10 mg/kg for Ca, 3.75 mg/kg for Fe, 5 mg/kg for Mg and Na, and 0.05 mg/kg for Pb. The methods for Fe and Pb analysis were validated using the IAEA-407 certified reference material (CRM; fish tissue) (International Atomic Energy Agency, Austria). The analysis including blank, CRM, and samples was performed in triplicate. CRM was repeated as every 10th sample. Mean recoveries for Fe and Pb were 108% and 91%, respectively. The relative standard deviations (RSD) used to evaluate the precision of the method were 5.9% for Fe and 8.2% for Pb. There was no available CRM for Ca, Mg, and Na at the time, and since they are ubiquitous to living tissue, we could not perform spiking experiments to assess recovery. For Ca, Mg, and Na, the analysis including blank, control standard

solution, and samples was performed in triplicate. Control standard solution was repeated as every 10th sample. RSDs in analyzed tissue were < 10%, which was rated as satisfactory. The results of metal content are expressed in  $\mu\text{g/g}$  on the dry mass of tissue.

### Protein Content Determination

Protein content was determined in suspensions of cell animal tissues and phosphate buffered saline (PBS, pH 7.4, 7.5 mM  $\text{Na}_2\text{HPO}_4$ , 2.5 mM  $\text{NaH}_2\text{PO}_4$ , 145 mM NaCl (Trevigen, Gaithersburg, MD, USA) according to Bradford's method, with bovine serum albumin (BSA; Albumin Fraktion V, > 98% pulv. bovine, Sigma, St. Louis, MO, USA) used as a standard [21]. The cell pellet obtained by successive centrifugation (twice at  $1,000 \times g/10 \text{ min}/+4 \text{ }^\circ\text{C}$ ; HETTICH Rotina 420R, Tuttlingen, Germany) was suspended in buffer and centrifuged at  $10,000 \times g/10 \text{ min}/+4 \text{ }^\circ\text{C}$  to obtain a clear cytosolic tissue extract. In 50  $\mu\text{L}$  of supernatant diluted 1:50, 1500  $\mu\text{L}$  of Bradford reagent was added. After 30 min, the absorbance was measured on an Infinite F200 microplate reader (Infinite F200, Tecan, Mannedorf, Switzerland) with Magellan software, at a wavelength of 595 nm, compared to the blank. Protein concentration was determined using a calibration curve made from different dilutions of the BSA. All analyses were done in triplicate. Due to the nonlinear dependence of the absorbance and standard concentrations, protein concentration was determined using a calibration curve obtained by nonlinear regression analysis. The protein concentration is expressed in mg protein/mL supernatant.

### Determination of Nonenzymatic Antioxidants in Hare Tissues

#### Total Glutathione (GSH) Determination

The tissue lysate for total GSH determination was prepared by homogenizing tissue using an Ultra-Turrax homogenizer (Labortechnik, Staufen, Germany) in 5% meta-phosphoric acid (Sigma-Aldrich, Taufkirchen, Germany) that served as a protein precipitator. The homogenate was centrifuged ( $12,000\text{--}14,000 \times g/10\text{--}15 \text{ min}/+4 \text{ }^\circ\text{C}$ ; HETTICH Rotina 420R, Tuttlingen, Germany), and the clear supernatant was taken and stored at  $-80 \text{ }^\circ\text{C}$  prior to analysis. The GSH concentration in the tissue homogenate was determined using a commercially available HT Glutathione Assay Kit (Trevigen®, Helgerman Ct, Gaithersburg, USA) [22, 23]. Serial dilutions of GSSG (oxidized form of glutathione) standard (50  $\mu\text{L}$ ), 50  $\mu\text{L}$  assay buffer (blank), and sample (50  $\mu\text{L}$ ), all previously diluted with buffer in the appropriate ratio, were added to wells containing 50  $\mu\text{L}$  of assay buffer. Immediately after adding the reaction mixture (150  $\mu\text{L}$ ), the absorbances of the standard, samples, and blank were read on a BIO-TEK

EL808IU microplate reader (BIO-TEK Instrument, Winooski, VT, USA) using the KC Junior software at a wavelength of 405 nm for 10 min. All measurements were made in triplicate. The results are expressed as nmoles of GSSG (equivalent to the total GSH) per milligram of protein (nmol/mg protein).

### Determination of Enzymatic Antioxidants in Hare Tissues

#### Glutathione Reductase (GR), Glutathione Peroxidase (GPx), Glutathione-S-Transferase (GST), and Superoxide Dismutase (SOD) Activity Assays

GR, GPx, GST, and SOD enzyme activity assays were performed in a 25% organ tissue homogenate prepared in PBS. The pellet obtained by successive centrifugation (twice at  $1,000 \times g/10 \text{ min}/+4 \text{ }^\circ\text{C}$ ) was suspended in 20% Triton X-100 buffer (Sigma-Aldrich, Taufkirchen, Germany). 0.4 mM phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich, Taufkirchen, Germany) was added as a protein proteases inhibitor, and then the cell suspension was centrifuged ( $10,000 \times g/10 \text{ min}/+4 \text{ }^\circ\text{C}$ ) to obtain a clear cytosolic tissue extract where the activity of GR, GPx, GST, and SOD was determined.

The catalytic activity of GR was determined using the commercial HT Glutathione Reductase Assay Kit (Trevigen®, Helgerman Ct, Gaithersburg, USA) [24]. GR restores intracellular GSH by reducing GSSG in the presence of NADPH. The serial dilutions involving GR standard (50  $\mu\text{L}/\text{well}$ ), samples (50  $\mu\text{L}/\text{well}$ ), and GR control (50  $\mu\text{L}$  GR buffer/well) with GR buffer in the appropriate ratio were prepared. A "Master-mix" (100  $\mu\text{L}/\text{well}$ ) was added to all wells, and immediately after initiating the reaction (50  $\mu\text{L}$  of NADPH solution per well), the absorbance was measured on a BIO-TEK EL808IU microtiter plate reader (BIO-TEK Instrument, Winooski, VT, USA) with KC Junior software, at a wavelength of 340 nm. The decrease in absorbance was monitored at 1-min intervals for 10 min at room temperature ( $25 \text{ }^\circ\text{C}$ ). All measurements were made in triplicate. The results are expressed as milliunits of GR per milligram of protein (mU/mg). One unit of catalytic GR activity represents the amount of enzyme that catalyzes 1  $\mu\text{mol}$  of GSSG per min per mg of extracted protein at pH 7.5 and at  $25 \text{ }^\circ\text{C}$ , which is equivalent to the oxidation of 1  $\mu\text{mol}$  of NADPH per minute under the same conditions.

HT Glutathione Peroxidase Assay Kit (Trevigen®, Helgerman Ct, Gaithersburg, USA) was used for the determination of GPx catalytic activity [25]. The reaction catalyzed by GPx is associated with the reaction that takes place under the action of the enzyme GR, which is the basis for the indirect determination of the catalytic activity of GPx. In wells of the microtiter plate, 140  $\mu\text{L}$  of assay buffer, 20  $\mu\text{L}$  of the reaction

mixture, 20  $\mu\text{L}$  of bovine serum GPx used as a positive control, and 20  $\mu\text{L}$  of sample were added in appropriate dilution. Wells, which represented control sample instead samples, contain the same volume of assay buffer. The reaction was initiated by adding 20  $\mu\text{L}$  of cumene hydroperoxide, and the absorbance was immediately measured on a microtiter plate reader at 340 nm for 10 min at room temperature (25 °C) at 30-s intervals. All measurements were made in triplicate. The catalytic activity of GPx is expressed in milliunits of enzyme per milligram of protein (mU/mg). One unit catalyzes the oxidation of 1 nmol NADPH to NADP<sup>+</sup> per min per mg of extracted protein at 25 °C and pH 7.0.

The catalytic activity of GST (cytosolic and microsomal) was determined using the commercial Glutathione-S-Transferase Assay Kit (Cayman®, Ann Arbor, MI, USA) following the 1-chloro-2,4-dinitrobenzene (CDNB)-reduced glutathione conjugation reaction [26]. In wells of the microtiter plate, the appropriate dilution of 150  $\mu\text{L}$  of assay buffer, 20  $\mu\text{L}$  of GSH, and 20  $\mu\text{L}$  of sample was added sequentially, while 20  $\mu\text{L}$  of horse liver GST was used as a positive control. Wells containing the same volume of assay buffer which was used instead of samples represented the control of GST activity. Immediately after adding 10  $\mu\text{L}$  CDNB, absorbance was measured at a wavelength of 340 nm. The increase in absorbance was monitored at 1-min intervals for 5 min at room temperature (25 °C). All measurements were made in triplicate. One unit of GST activity is defined as the amount of enzyme that catalyzes the reduction of 1 nmol CDNB per minute per milligram of the extracted protein at pH 6.5 at 25 °C. GST activity in the samples is expressed in mU/mg protein.

The commercial HT Superoxide Dismutase Assay Kit (Trevigen®, Helgerman Ct, Gaithersburg, USA) was used for the determination of SOD catalytic activity [27]. SOD catalytic activity is based on measuring the percent inhibition of the formation rate of quaternary ammonium salts (WST-1) upon superoxide anion radicals ( $\text{O}_2^-$ ) generation in the xanthine/xanthine oxidase system (WOD). In the analysis of serial dilutions of the SOD standard (25  $\mu\text{L}$ /well) as a positive control, serial dilutions of samples (25  $\mu\text{L}$ /well) and controls of SOD (25  $\mu\text{L}$  SOD buffer/well instead of samples) were prepared. A “Master-mix” (150  $\mu\text{L}$ /well) was added to all wells, and immediately after initiating the reaction with 25  $\mu\text{L}$  of xanthine solution/well, the absorbance was measured on a BIO-TEK EL808IU microtiter plate reader with KC Junior software at a wavelength of 450 nm. The absorbance decrease was monitored at 1-min intervals for 10 min at room temperature (25 °C). All measurements were made in triplicate. Since the dependence of the percent absorbance inhibition and the amount of protein in the sample was nonlinear, SOD activity was determined by nonlinear regression analysis using GraphPad 5.0 software (GraphPad Software, Inc., 1992–2007, San Diego, CA, USA). The amount of

protein in the sample that causes inhibition of 50% in color formation at 25 °C and pH 8.5 represents one international unit of SOD activity (U/mg protein). The catalytic activity of SOD is expressed in units of enzyme per milligram of protein (U/mg).

### Determination of Lipid Peroxidation (LPO) Products in Hare Tissues

LPO was detected in tissue homogenate prepared by 25 mg of tissue and 250  $\mu\text{L}$  of RIPA buffer (RadioImmuno Precipitation Assay, Sigma-Aldrich, Taufkirchen, Germany) with PMSF as proteases inhibitor (Sigma-Aldrich, Taufkirchen, Germany). Homogenate samples were sonicated and centrifuged (1,600  $\times g$ /10 min/+ 4 °C; Jouan MR18.12/22 High speed benchtop centrifuges, Jouan, France), and the supernatant was used for LPO analysis.

The determination of LPO products was based on the reaction of malondialdehyde (MDA) and thiobarbituric acid (TBA) and was conducted by the commercial TBARS Assay Kit (Cayman®, Ann Arbor, MI, USA) [28]. LPO in sample tissue homogenates was determined by measuring thiobarbituric reactive substance (TBARS). In 100  $\mu\text{L}$  of the sample, 100  $\mu\text{L}$  of SDS (sodium dodecyl sulfate) and 4 mL of colored reagent prepared from TBA, acetic acid, and sodium hydroxide were added according to the manufacturer’s instructions. The reaction was processed at a high temperature (95 °C) using a Julabo TW8 water bath (Julabo Labortechnik GmbH, Germany) for 60 min. Then, the mixture was cooled on ice for 10 min to stop the reaction and centrifuged (Jouan MR18.12/22 High speed benchtop centrifuges, Jouan, France) at 1,600  $\times g$ /10 min at 4 °C. The concentration of TBARS was measured colorimetrically on a microtiter plate reader (Infinite F200, Tecan, Mannedorf, Switzerland) with Magellan software at a wavelength of 530 nm. MDA was used as a standard. All measurements were made in triplicate. The results are expressed in nmol of MDA/g of wet tissue.

### Statistical Analysis

Descriptive statistical analyses as well as all other statistical calculations were performed using the commercial software Statistica® v. 13.0 (StatSoft Inc., Tulsa, OK, USA) at the  $p < 0.05$  level of significance. All assays and analyses were performed at least in triplicate, and the results are expressed as the median with minimal and maximal values. Differences between the experimental and control groups in metal content and enzyme activity in each hare organ were tested by the nonparametric Mann–Whitney U test. For correlation analysis between metal content and enzymes in all hare organs, nonparametric Kendal-Tau, multiple regression, and

principal component analysis (PCA) statistical tests were used.

## Results and Discussion

The ionic mechanism of lead toxicity can be explained in several ways, but one of the most mentioned is its ability to substitute bivalent (Ca, Fe, and Mg) or monovalent (Na) cations through calcium channels, resulting in less effective regulation of channel closure and higher cell permeability to lead [17, 29]. This change in the ionic mechanism has a direct negative effect on a number of biological processes, such as cellular signaling, apoptosis, protein synthesis, and neurotransmitter release. Neurological disorders occur when toxic lead crosses the blood–brain barrier and then accumulates in astroglial cells, causing damage and interfering with the formation of the myelin sheath.

To explain the mechanism of ion substitution due to lead intoxication, the contents of metals (Ca, Fe, Mg, Na, and Pb), total glutathione content (GSH), lipid peroxidation level (LPO), and activities of antioxidant enzymes (GPx, glutathione peroxidase; GR, glutathione reductase; GST, glutathione-S-transferase; and SOD, superoxide dismutase) in hares were analyzed as very important bioindicators of toxicity. Table 1 gives the contents of Ca, Fe, Mg, and Na ( $\mu\text{g}$  of metal/g of dry organ mass) in hare organs in the experimental and control groups, showing the median values, minimum and maximum, and level of significance.

In the control group of hares, we observed the highest contents of Ca and Na in muscle and the highest contents of Fe and Mg in the liver. A slightly different pattern was

observed in the experimental group, where the highest values of Ca and Mg were found in the brain and of Na in the kidney. The metal contents in the liver and muscle of the experimental group were lower than in the control group. Statistically significant decreases in Fe (64.6%;  $p=0.0099$ ) and Mg (24.5%;  $p=0.0179$ ) contents occurred in the liver, while major reductions in Ca (65.0%;  $p=0.0103$ ) and Na (48.5%;  $p=0.0129$ ) contents were observed in the muscle. Simultaneously, in the brain and kidney, contents of Ca and Na increased in comparison with the control group with a significant change only in Ca content (48.0%,  $p=0.0122$  in the kidney and 324.5%,  $p=0.0323$  in the brain). Similarly, as in the liver and muscle, there were evident but insignificant decreases in Fe content (33.1% in the kidney and 41.9% in the brain).

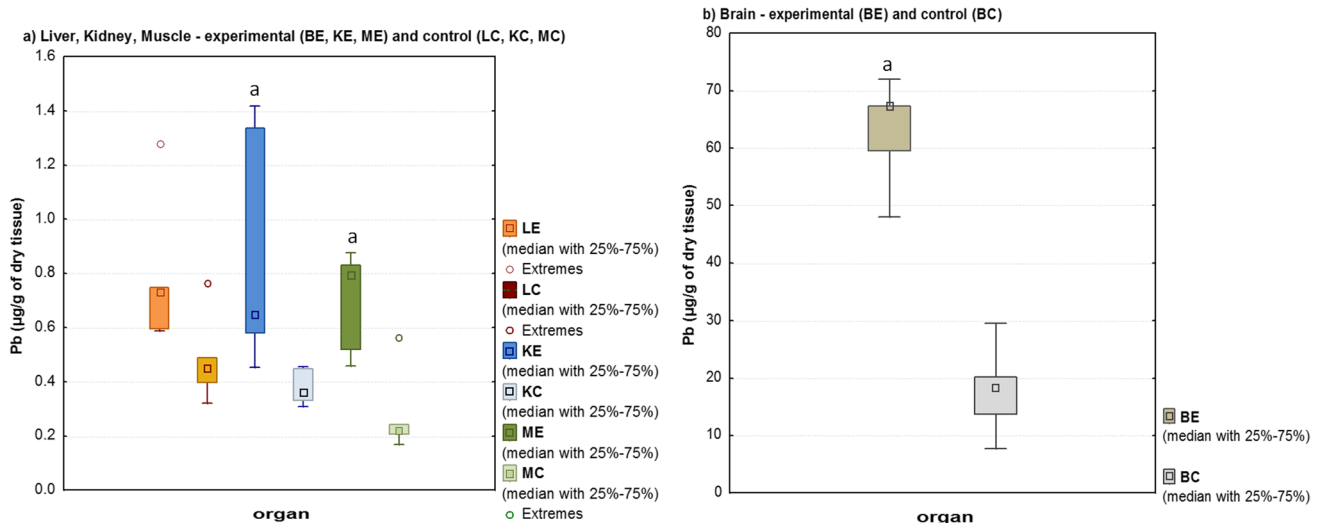
All of these results might mean that lead either mobilizes intracellular cation stores or acts as a calcium substitute. No studies on hares have been found in the literature that has addressed the ionic status associated with lead toxicity as a bioindicator. However, a study conducted by Dobrakowski et al. [30] on humans showed similar results to those obtained in this study. Namely, after short-term exposure to lead, calcium levels significantly increased, which could be related to the competitive displacement of calcium from binding sites in cells intoxicated by lead. Also the elevation of Mg could be also caused by the increased release of Mg from tissues due to its displacement from binding sites by lead ions.

The lead contents in hare organs are presented in Fig. 2a,b (all values are given in  $\mu\text{g}$  Pb per g of dry organ mass). It is evident that the accumulation of lead was higher in all hare tissues of the experimental group than in the tissues of the

**Table 1** Metal content (Ca, Fe, Mg, and Na;  $\mu\text{g}$  of metal/g of dry tissue mass) in hare organs in the experimental ( $N=5$ ) and control groups ( $N=5$ )

Metal	Treatment	Metal content ( $\mu\text{g}/\text{g}$ of dry tissue mass)			
		Liver	Kidney	Muscle	Brain
Ca	Experimental	173.78 (162.01–252.05)	359.02 <sup>a</sup> (0.0122) (326.03–469.62)	190.96 <sup>a</sup> (0.0103) (123.62–268.1294)	1424.16 <sup>a</sup> (0.0325) (404.67–3395.54)
	Control	207.12 (172.02–233.61)	242.59 (215.35–257.79)	544.96 (329.49–1516.05)	335.49 (243.86–805.28)
Fe	Experimental	307.91 <sup>a</sup> (0.0099) (241.02–431.09)	345.95 (223.56–668.43)	95.14 <sup>a</sup> (0.0145) (67.03–106.25)	97.49 (4.51–213.99)
	Control	868.69 (734.68–1027.63)	517.11 (445.10–550.59)	144.63 (107.60–221.94)	167.94 (76.15–262.69)
Mg	Experimental	402.20 <sup>a</sup> (0.0179) (313.44–467.37)	496.76 (316.65–786.46)	612.87 <sup>a</sup> (0.0367) (549.56–697.32)	452.46 (348.17–498.87)
	Control	532.38 (474.21–562.71)	498.03 (465.40–520.56)	714.47 (640.54–762.39)	405.01 (192.86–434.87)
Na	Experimental	1975.57 (1871.95–2590.03)	3334.70 (2150.41–4419.73)	1151.66 <sup>a</sup> (0.0129) (861.35–1552.62)	3081.12 (1739.12–3460.63)
	Control	2014.88 (1796.88–2364.27)	2648.24 (2289.27–2973.35)	2236.54 (1609.08–2591.79)	2904.22 (1653.31–3125.88)

The results are presented as median, minimum, and maximum values. Median values marked with lowercase letter *a* represent significant differences (corresponding  $p$  values are in parentheses in italics) in the experimental and control groups ( $p < 0.05$ ; nonparametric Mann–Whitney U test)



**Fig. 2** Lead content (all values are given in µg Pb per g of dry tissue mass) in hare organs: **a)** L, liver; K, kidney; M, muscle; and **b)** B, brain in the experimental (E;  $N=5$ ) and control (C;  $N=5$ ) groups. The results are presented as median, minimum, and maximum values.

Median values marked with lowercase letter *a* represent significant differences between the experimental and control groups ( $p < 0.05$ ; nonparametric Mann–Whitney U test)

control group with the most potency in the brain (266.6%;  $p = 0.0086$ ) (Fig. 2b) and then decreased in the following order: muscle (264.7%;  $p = 0.0367$ ), kidney (78.2%;  $p = 0.0216$ ), and liver (63.3%) (Fig. 2a).

The direct impact of environmental pollution on hare survival was previously investigated in a study by Wajdzik et al. [5], who found that lead mainly accumulated in the brain. The content of toxic lead has also been studied by Pedersen and Lierhagen [31]. Their findings showed some different patterns, with low accumulation in the muscle and high accumulation in the liver and kidney. The minimum concentration of Pb in liver presented in this study was similar to the range previously reported by Amuno et al. [32]. These similar findings confirm that differences in the degree of accumulation in various organs occurred because lead tends to accumulate mainly in the brain or to be transported to the liver and kidney for storage and detoxification. Some other studies reported significantly higher accumulation of lead in liver and kidney of brown hare due to the contamination of plants by aerogenous emissions [33]. The variability between organs clearly illustrates the difficulties in making relevant comparisons between species and experiments. Also, factors such as nutrition, region, and living conditions are involved [34]. One of the main pathogenic effects of lead is a direct effect on enzyme activities that competitively inhibits the absorption of important trace minerals and deactivates the antioxidant sulfhydryl pools [35]. Our study of long-term exposure to a low level of environmental lead revealed a difference in enzyme activities in target hare organs. Enzyme systems are involved in the neutralization of reactive oxygen species, representing a major defense system

against lead-induced toxicity. The total GSH and LPO contents and the activities of antioxidant enzymes (GPx, GR, GST, and SOD) in the experimental and control groups in hare organs are presented in Table 2. Organ resistance to oxidative stress due to long-term lead exposure was determined by estimating the total GSH content, which includes reduced GSH and oxidized GSSG forms. Glutathione (GSH) is a tripeptide with sulfhydryl groups and is found in mammalian tissues at millimolar concentrations. Its main role as an antioxidant is binding with free radicals generated under the influence of lead [36].

Our results showed significantly higher GSH values only in the liver (25.5%,  $p = 0.0145$ ) and in the kidney (69.1%,  $p = 0.0216$ ) of the experimental groups compared with the control group. As previously mentioned by Gurer and Ercal, GSH is a tripeptide containing cysteine that has a reactive SH group with reductive potency and plays a vital role in the protection of cells against oxidative stress. The mechanism of toxicity results in binding  $Pb^{+2}$  exclusively to the SH group, which decreases the GSH levels and can interfere with its antioxidant activity [37]. Under stress conditions, GSH is utilized, resulting in the formation of its oxidized GSSH form. To determine the ability to convert GSSH back to the GSH form, GR activity was estimated. The activity of GR in the liver and kidneys increased compared with that in the control group, while in the muscle tissue, it significantly decreased (69.6%,  $p = 0.0086$ ), which indicates an increased need for conversion of the oxidized form to the reduced form of GSH and was also observed in a study by Sandhir et al., where the results on lead-exposed rats showed that inhibition of GR is responsible for altered GSH status. Consequently,

**Table 2** Total glutathione content (GSH; nmol/mg protein), lipid peroxidation level (LPO; nmol MDA/g of wet organ mass), and activities of antioxidant enzymes (GPx glutathione peroxidase, GR glutathione reductase, GST glutathione-S-transferase, and SOD superoxide dismutase) in hare organs in the experimental ( $N=5$ ) and control ( $N=5$ ) groups

Variable	Treatment	Content/enzyme activity			
		Liver	Kidney	Muscle	Brain
GSH (nmol/mg protein)	Experimental	62.43 <sup>a</sup> (0.0145) (61.64–69.65)	35.63 <sup>a</sup> (0.0216) (30.35–40.46)	13.55 (10.19–20.35)	21.73 (20.02–24.35)
	Control	49.75 (43.55–52.37)	21.07 (20.33–31.66)	11.65 (10.56–13.93)	20.54 (17.35–25.33)
LPO (nmol MDA/g tissue)	Experimental	24.31 <sup>a</sup> (0.0186) (22.37–39.90)	26.42 (24.16–27.76)	21.37 (21.29–21.91)	20.58 (20.03–21.28)
	Control	20.31 (17–97–21.87)	20.12 (11.72–27.13)	21.26 (21.02–21.93)	21.89 (21.59–21.92)
GPx (mU/mg protein)	Experimental	215.14 <sup>a</sup> (0.0223) (211.53–228.05)	227.65 (221.73–228.85)	211.62 (209.90–214.73)	201.69 (198.46–202.66)
	Control	206.06 (205.06–210.47)	228.45 (222.47–228.65)	205.66 (94.26–216.15)	203.57 (201.69–204.93)
GR (mU/mg protein)	Experimental	32.90 (15.58–53.35)	38.64 (31.48–42.68)	7.40 <sup>a</sup> (0.0086) (1.88–7.86)	32.56 (30.80–37.38)
	Control	26.25 (9.58–41.66)	32.49 (31.24–38.28)	24.35 (8.90–43.57)	37.28 (32.84–46.67)
GST (mU/(mg protein)	Experimental	20.65 <sup>a</sup> (0.0101) (18.67–23.79)	150.72 (30.71–172.02)	183.44 (166.93–236.32)	193.81 (134.07–235.54)
	Control	67.36 (33.67–93.75)	173.66 (122.98–186.06)	202.37 (155.98–222.11)	240.88 (101.97–276.90)
SOD (U/mg protein)	Experimental	6.76 <sup>a</sup> (0.0200) (5.93–8.77)	3.71 <sup>a</sup> (0.0243) (3.54–3.92)	9.02 (7.32–11.65)	5.66 (2.23–6.35)
	Control	4.21 (3.34–4.67)	2.52 (2.49–2.98)	8.02 (6.22–9.33)	6.06 (5.25–7.14)

The results are presented as median, minimum, and maximum values. Median values marked with lowercase letter *a* represent significant differences (corresponding *p* values are in parentheses in italics) in the experimental and control groups ( $p < 0.05$ ; nonparametric Mann–Whitney *U* test)

enhanced peroxidation of membrane lipids is accompanied by decrease in the antioxidant capacity of the cell [38]. The activity of SOD, an antioxidant enzyme presenting the first and second lines of defense against ROS cytotoxicity, significantly increased in the kidney (47.2%;  $p = 0.0243$ ) and liver (60.6%;  $p = 0.0200$ ) of the experimental group, while the activity in the brain was lower in the experimental than in the control group (7.1%). This behavior indicates that these organs reflect the greater need for the detoxification of superoxide anions, depleting cellular GSH and increasing the amounts of LPO products. The catalytic reaction between SOD and ROS formed hydrogen peroxide, whose cytotoxicity was balanced by GPx activity. The activity of GPx in the liver significantly increased (4.4%,  $p = 0.0223$ ) in comparison with the control group. Similar statements were reported by Limón-Pacheco and Gonsebatt in their study mentioning that GPx and SOD activities were higher in the liver than in the kidney and muscle indicating that GPx, GR, GST, and SOD appear to be organ-specific [39].

GST activity was also analyzed to estimate the detoxification efficiency of the LPO products in the organs. The results showed that GST activity in the liver of the experimental group of hares was more than three-fold lower than in the control groups ( $p = 0.0101$ ). A similar conclusion was reported in the study by Obolenskaya et al., where an imbalance in detoxification capacity of GST could be measured

as a result of increased production of PAH-DNA adducts. Their findings points to clear relationships between GR and GST activity indicating that GST activity may well be a common denominator in the regulation of glutathione levels and redox status [40]. Furthermore, the effects of LPO levels on the amounts of MDA in hare organs were assessed. Experimental animals exhibited a significantly higher level of MDA only in the liver (19.7%,  $p = 0.0186$ ), while in the kidney, a substantial but not statistically significant increase in LPO was recorded. However, the main effect of lead was an increased level of LPO that may not be a direct effect of lead but rather an indirect effect on free radical scavenging by other enzymes and glutathione. The progression of all the above-mentioned mechanisms makes the cell extremely vulnerable to oxidative stress [17].

Furthermore, to explain the ionic exchange mechanism in more detail and to evaluate the oxidative damage induced by lead accumulation in hare tissues, the influence of lead on metals (Ca, Fe, Mg, and Na), total glutathione content (GSH), lipid peroxidation level (LPO), and activities of antioxidant enzymes (GPx, GR, GST, and SOD) in the experimental group was studied by the Kendall-Tau correlation analysis. Correlation analyses of pooled data ( $N = 20$ ; five animals  $\times$  four organs) indicated the strong positive correlation ( $\tau = 0.45$ ) between lead and Ca content ( $p = 0.0053$ , Table 3), while the correlation with Na was positive and



**Table 3** Coefficients of correlation between different metals (Ca, Fe, Mg, Na, and Pb) in the experimental group (N=20; five animals × four organs) represented by the nonparametric Kendall-Tau correlation test

Metal	Ca	Fe	Mg	Na	Pb
Ca	1.00				
Fe	-0.12 (0.4754)	1.00			
Mg	-0.17 (0.2992)	-0.17 (0.2992)	1.00		
Na	<b>0.42</b> (0.0094)	<b>0.34</b> (0.0379)	0.05 (0.7456)	1.00	
Pb	<b>0.45</b> (0.0053)	-0.22 (0.1730)	-0.04 (0.7952)	0.25 (0.1194)	1.00

Statistically significant correlations ( $p < 0.05$ ) are presented with bold type numbers and with corresponding  $p$  values (written in parentheses in italics)

moderate ( $\tau = 0.25$ ) but not significant. The correlation of lead content with Fe was negative and moderate ( $\tau = -0.22$ ), while with Mg, it was very weak ( $\tau = -0.04$ ). However, neither correlations were statistically significant.

The Kendall-Tau correlation revealed a very weak influence of lead on the enzyme systems. Therefore, a positive weak ( $\tau = 0.15$ ) correlation between lead content and GST activity was found (Table 4), while correlations with other enzyme systems were negative, with a strong impact on GPx activity ( $\tau = -0.31$ ) and a moderate impact on LPO content ( $\tau = -0.31$ ). Correlations between different enzyme systems indicated that GSH was significantly positively correlated with GR activity ( $\tau = 0.49, p = 0.0045$ ) and LPO content ( $\tau = 0.42, p = 0.0041$ ), as was GPx with LPO content ( $\tau = 0.61, p = 0.0004$ , Table 4), while significant negative correlations were found between GSH and GST activity ( $\tau = -0.66, p = 0.0009$ ), GR and SOD activity ( $\tau = -0.42, p = 0.0138$ ), and GST and LPO content ( $\tau = -0.37, p = 0.0308$ ).

Many studies have shown significant changes in GSH levels in animals [41] or humans [42] after exposure to a low level of lead. However, in our study, GSH failed to show a strong positive correlation with tissue lead levels. Some previously published data suggests that exposure to lead can result in an increase or decrease in GSH levels depending on the concentration of exposed lead, duration of lead exposure, and magnitude of oxidative stress inside the cell [37]. In addition to nonparametric tests, a multiple linear regression

test was used. It simultaneously compares changes in different parameters, in this case, metals and enzyme systems, during exposure to lead, helping to create a linear predictive model for intoxication with lead. Multiple linear regression resulted in the following correlations, where bold typeface indicates significant correlations with  $p < 0.05$ :

$$Pb_{content} = 3.63 + 0.01 \times Ca_{content} - 0.03 \times Mg_{content} + 0.02 \times Na_{content} - 0.13 \times Fe_{content} (R^2 = 0.761; p < 0.00015)$$

$$Pb_{content} = 448.75 + 0.12 \times GSH_{content} + 0.45 \times GR_{activity} - 2.04 \times GPx_{activity} + 0.10 \times GST_{activity} - 3.75 \times SOD_{activity} - 0.14 \times LPO_{content}$$

$$(R^2 = 0.863; p < 0.0034)$$

Kendall-Tau correlation showed that the increasing content of lead in organs caused stronger exchange of Ca and Na metals, which was confirmed by the multiple regression analysis. Furthermore, nonparametric analysis showed a weak influence of lead on the enzyme systems, but multiple linear regression equations suggested that GPx and SOD were predominantly influenced by long lead exposure.

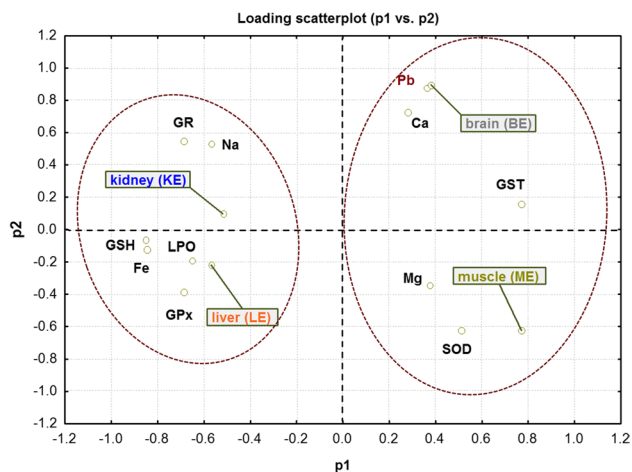
Finally, to determine the effect of long-term lead exposure on metal metabolism and on enzymatic activity in the

**Table 4** Coefficients of correlation between Pb and total glutathione content (GSH), lipid peroxidation level (LPO), and activities of antioxidant enzymes (GPx glutathione peroxidase, GR glutathione reduc-

tase, GST glutathione-S-transferase, and SOD superoxide dismutase) in the experimental group (N=20; five animals × four organs) represented by nonparametric Kendall-Tau correlation test

Metal/enzyme	Pb	GSH	LPO	GPx	GR	GST	SOD
GSH	-0.15 (0.3837)	1.00					
LPO	-0.28 (0.3778)	<b>0.42</b> (0.0045)	1.00				
GPx	-0.31 (0.0750)	0.27 (0.1204)	<b>0.61</b> (0.0004)	1.00			
GR	-0.03 (0.8498)	<b>0.49</b> (0.0045)	0.20 (0.2403)	0.28 (0.1034)	1.00		
GST	0.15 (0.3837)	- <b>0.66</b> (0.0009)	- <b>0.37</b> (0.0308)	-0.27 (0.1204)	-0.31 (0.0750)	1.00	
SOD	-0.12 (0.4717)	-0.15 (0.3837)	-0.25 (0.1396)	-0.23 (0.1849)	- <b>0.42</b> (0.0138)	0.02 (0.9095)	1.00

Statistically significant correlations ( $p < 0.05$ ) are presented with bold type numbers and with corresponding  $p$  values (written in parentheses in italics)



**Fig. 3** Principal component analysis (PCA) applied to metals (Ca, Fe, Mg, Na, and Pb), total glutathione content (GSH), lipid peroxidation level (LPO), and activities of antioxidant enzymes (GPx, glutathione peroxidase; GR, glutathione reductase; GST, glutathione-S-transferase; and SOD, superoxide dismutase) in hare organs in the experimental group ( $N=20$ ; five animals  $\times$  four organs)

**Table 5** Principal component analysis (PCA) eigenvalues with the analysis of the sum of variance for metals (Ca, Fe, Mg, Na, and Pb) and total glutathione content (GSH), lipid peroxidation level (LPO), and activities of antioxidant enzymes (GPx, glutathione peroxidase, GR, glutathione reductase, GST, glutathione-S-transferase, and SOD superoxide dismutase) in hare organs in the experimental group ( $N=20$ ; five animals  $\times$  four organs). The number of components is six

Component	Eigenvalue	Total variance (%)	Cumulative eigenvalue	Cumulative (%)
1	5.66	37.76	5.66	37.76
2	3.85	25.67	9.51	63.43
3	2.87	19.14	12.39	82.57
4	0.88	5.88	13.27	88.45
5	0.75	4.98	14.02	93.44
6	0.46	3.07	14.48	96.51

**Table 6** Eigenvector and loading values for variables: metals (Ca, Fe, Mg, Na, and Pb), total glutathione content (GSH), lipid peroxidation level (LPO), and activities of antioxidant enzymes (GPx, glutathione peroxidase, GR, glutathione reductase, GST, glutathione-S-transferase, and SOD superoxide dismutase) in hare organs in the experimental ( $N=20$ ; five animals  $\times$  four organs) group in the first three main components

Variable	Eigenvector spreadsheet			Loading spreadsheet		
	Component 1	Component 2	Component 3	Component 1	Component 2	Component 3
Ca	0.12	0.37	-0.10	0.31	0.71	-0.18
Fe	-0.35	-0.06	0.16	-0.85	-0.10	0.27
Mg	0.16	-0.18	0.39	0.36	-0.35	0.67
Na	-0.24	0.27	0.29	-0.55	0.54	0.50
Pb	0.15	0.45	-0.07	0.39	0.87	-0.12
GSH	-0.36	-0.03	-0.28	-0.85	-0.05	-0.47
LPO	-0.27	-0.10	-0.05	-0.66	-0.17	-0.09
GPx	-0.29	-0.20	0.28	-0.70	-0.37	0.47
GR	-0.29	0.28	0.01	-0.67	0.56	0.02
GST	0.32	0.08	0.31	0.77	0.13	0.53
SOD	0.22	-0.32	-0.25	0.49	-0.63	-0.43

hare organs, principal component analysis (PCA) was used. This analysis, in addition to determining the factor coordinates of variables and cases, gives a fairly good idea of the variance that can be explained by a given number of factors. Figure 3 presents the values of all main components together with their contributions to the total variance.

The number of main components implemented in PCA was estimated by the Kaiser-Guttman method and by the Cattell scree test. According to the Kaiser-Guttman method, principal components (p) with a value greater than 1 are retained in the analysis. It can be seen from the values in Table 5 that the three main components (p1, p2, and p3) should be used in PC analysis. The first component explains 37.76% of the total variance, the second explains 25.67%, and the third explains 19.14%. From the Cattell scree test, it is evident that the eigenvalues with the largest contribution to the total variance have the smallest number (p1) and eigenvalues with values greater than 1 should be used in PCA. Thus, it can be concluded that the analyzed parameters of long-term lead exposure can be described by three main components.

For the interpretation of the main components, eigenvectors were used. Their values are presented in Table 6 and are relatively evenly distributed in the first main component across all variables. In the second and third main components, there is a greater disproportion, which allows the separation of the original variable with stronger participation and helps to explain and summarize the overall variability.

Table 6 presents the grouping of the analyzed variables around each main component. Most of the parameters used in the analysis of long-term lead exposure may be explained by the main component p1 (high correlation of observed variables and the main component p1). High positive correlation of the p1 component was observed with GST (0.77), and negative correlations were observed with Fe (-0.85), Na (-0.55), GSH (0.85), GR (-0.67), GPx (-0.70), GST (-0.77), and LPO (-0.66), while variables Ca, lead, and

SOD were grouped around the p2 component. Figure 3 shows the distribution of the analyzed parameters in the hare organs in all four quadrants. Parameters analyzed in the brain and muscle proved to be more significant in the analysis of lead toxicity determination, and all parameters according to PCA were grouped on the right side of the diagram (in the first and second quadrants), as opposed to variables analyzed in the liver and kidney, which were in grouped in the third and fourth quadrants.

Using PCA, it was possible to determine which of the parameters had a dominant effect on lead-induced toxicity. According to the data obtained by the first main component (p1), p1 can explain 37.76% of all interactions in the observed data, with the main influencing factors being Fe, GSH, and GST. The second main component explains 25.67% of the variances of the observed data with dominating parameters Ca, lead, and SOD. A total of 63.43% of the variance can be explained by the two main components.

## Conclusions

Lead concentrations were significantly higher in the brain, muscle, and kidney of hares from the exposed study area than in the tissues of hares in the control group. Lead affects mammalian systems by directly interfering with oxidative enzyme status and generating ROS, which results in critical damage to enzymes and simultaneously impairs the antioxidant defense system. This study indicates that lead can promote a significant increase in LPO, leading to oxidative stress in the hare liver. Additionally, other main oxidative stress enzymes showed increases in the liver (GSH, SOD, GR, and GPx) in addition to decreased activity levels of GST. Competitive displacement of calcium from its binding sites in cells by lead has also been detected. This study confirmed that the European hare, with its settled life, may be used as a bioindicator of environmental pollution with lead and probably other metals. The obtained results suggest that lead, at relatively low environmentally relevant concentrations, can have a negative impact on oxidative status in hares, making their survival and/or population vulnerable. Furthermore, work is needed to find effective and safe interventions for lowering lead exposure to the environment and wildlife and consequently to humans.

**Author Contribution** Željko Linšak: Investigation, formal analysis, data curation, and writing—original draft preparation.

Ivana Gobin: Validation and reviewing and editing.

Dijana Tomić Linšak: Conceptualization, methodology, formal analysis, data curation, writing, reviewing and editing, and supervision.

Dalibor Broznić: Formal analysis, data curation, software, visualization, writing, and reviewing and editing.

All authors read and approved the final draft of the manuscript.

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**Data Availability** We agree to full transparency of the data if needed. Raw data will be provided on request.

## Declarations

**Ethics Approval** The animals were hunted during the regular hunting period in Croatia, in compliance with the Law on Hunting taken from the European Union Directive, and donated by the hunters, so no approval of research ethics committees was required to accomplish the goals of this study.

**Conflict of Interest** The authors declare no competing interests.

## References

- Lupino-Gratão P, Vara Prasad NM, Cardoso FP, Lea PJ, Azevedo AR (2005) Phytoremediation: green technology for the clean-up of toxic metals in the environment. *Braz J Plant Physiol* 17(1):53–64. <https://doi.org/10.1590/S1677-04202005000100005>
- Reimann C, Fabian K, Birke M, Filzmoser P, Demetriades A, Négrel P, Oorts K, Matschullat J, de Caritat P, The GEMAS Project Team (2017) Establishing geochemical background and threshold for 53 chemical elements in European agricultural soil. *J Appl Geochem*. <https://doi.org/10.1016/j.apgeochem.2017.01.021>
- Peh Z (2009) Statistical processing. In: Halamić J, Miko S (eds) *Geochemical Atlas of the Republic of Croatia*. Croatian Geological Survey, Zagreb, pp 68–69
- Špirić Z, Srebočan E, Prevendar Crnić A (2012) Mercury in hares organs (*Lepus europaeus* Pallas) in the vicinity of the mercury-contaminated natural gas treatment plant in Croatia. *J Environ Sci Health* 47(1):77–83. <https://doi.org/10.1080/10934529.2012.629584>
- Wajdzik M, Halecki W, Kalarus K, Gašiorek M, Pająk M (2017) Relationship between heavy metal accumulation and morphometric parameters in European hare (*Lepus europaeus*) inhabiting various types of landscapes in southern Poland. *Ecotoxicol Environ Saf* 145:16–23. <https://doi.org/10.1016/j.ecoenv.2017.06.070>
- Reichlin T, Klansek E, Hackländer K (2006) Diet selection by hares (*Lepus europaeus*) in arable land and its implications for habitat management. *Eur J Wildl Res* 52:109–118. <https://doi.org/10.1007/s10344-005-0013-3>
- Sharma P, Dubey RS (2005) Lead toxicity in plants. *Braz J Plant Physiol* 17(1):35–52. <https://doi.org/10.1590/S1677-04202005000100004>
- Petrović Z, Teodorović V, Dimitrijević M, Borozan S, Beuković M, Milićević D (2013) Environmental Cd and Zn concentrations in liver and kidney of European hare from different Serbian regions: age and tissue differences. *Bull Environ Contam Toxicol* 90(2):203–207. <https://doi.org/10.1007/s00128-012-0901-7>
- Linšak Ž, Tomić Linšak D, Špirić Z, Srebočan E, Glad M, Milin Č (2013) Effects of mercury on glutathione and glutathione-dependent enzymes in hares (*Lepus europaeus* Pallas). *J Environ*

- Sci Health A 48(11):1325–1332. <https://doi.org/10.1080/10934529.2013.781869>
10. Tomić Linšak D, Linšak Ž, Špirić Z, Srebočan E, Glad M, Cenov A, Jakovac H, Milin Č (2014) Influence of cadmium on metallothionein expression and products of lipid peroxidation in the organs of hares (*Lepus europaeus* Pallas). *J Appl Toxicol* 34(3):289–295. <https://doi.org/10.1002/jat.2880>
  11. Kalia K, Flora SJ (2005) Strategies for safe and effective therapeutic measures for chronic arsenic and lead poisoning. *J Occup Health* 47(1):1–21. <https://doi.org/10.1539/joh.47.1>
  12. Poreba R, Gac P, Poreba M, Andrzejak R (2011) Environmental and occupational exposure to lead as a potential risk factor for cardiovascular disease. *Environ Toxicol Pharmacol* 31(2):267–277. <https://doi.org/10.1016/j.etap.2010.12.002>
  13. Farmand F, Ehdaie A, Roberts CK, Sindhu RK (2005) Lead-induced dysregulation of superoxide dismutase, catalase, glutathione peroxidase, and guanylate cyclase. *Environ Res* 98(1):33–39. <https://doi.org/10.1016/j.envres.2004.05.016>
  14. Wang J, Wu J, Zhang Z (2006) Oxidative stress in mouse brain exposed to lead. *Ann Occup Hyg* 50(4):405–409. <https://doi.org/10.1093/annhyg/mei079>
  15. Zhang YM, Liu XZ, Lu H, Mei L, Liu ZP (2009) Lipid peroxidation and ultrastructural modifications in brain after perinatal exposure to lead and/or cadmium in rat pups. *Biomed Environ Sci* 22(5):423–429. [https://doi.org/10.1016/S0895-3988\(10\)60021-9](https://doi.org/10.1016/S0895-3988(10)60021-9)
  16. Prasanthi RPJ, Devi CB, Basha DC, Reddy NS, Reddy GR (2010) Calcium and zinc supplementation protects lead (Pb)-induced perturbations in antioxidant enzymes and lipid peroxidation in developing mouse brain. *Int J Dev Neurosci* 28(2):161–167. <https://doi.org/10.1016/j.ijdevneu.2009.12.002>
  17. Flora G, Gupta D, Tiwari A (2012) Toxicity of lead: a review with recent updates. *Interdiscip Toxicol* 5(2):47–58. <https://doi.org/10.2478/v10102-012-0009-2>
  18. Singh PK, Nath R, Pal R, Dixit RK (2019) Environmental lead toxicity. book: *Heavy Metals Toxicity in Biological Systems*. Publisher, Discovery Publishing House Pvt. Ltd., New Delhi, India, pp 34–50
  19. (2019) Official Gazette of the Republic of Croatia, 140/2005 and 37/2019 Croatian Hunting Law; Ordinance on the manner of use of hunting weapons and ammunition. Croatian Parliament, Croatia 2005, Ministry of Agriculture, Croatia
  20. European Union (2013) Council Directive 92/43/EEC of the European Parliament and the Council of 21 May 1992 on the conservation of natural habitats and of wild fauna and flora. <https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:31992L0043&from=EN>. Accessed 31 Aug 2021
  21. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72(1–2):248–254. <https://doi.org/10.1006/abio.1976.9999>
  22. Marinić J, Broznić D, Milin Č (2016) Preexposure to olive oil polyphenols extract increases oxidative load and improves liver mass restoration after hepatectomy in mice via stress-sensitive genes. *Oxid Med Cell Longev* 2016:1–13. <https://doi.org/10.1155/2016/9191407>
  23. Onyango GI, Shaharyar KM (2006) Oxidative stress, mitochondrial dysfunction, and stress signaling in Alzheimer's disease. *Curr Alzheimer Res* 3(4):339–349. <https://doi.org/10.2174/156720506778249489>
  24. Dringen R, Gutterer JM (2002) Glutathione reductase from bovine brain. *Methods Enzymol* 348:281–288. [https://doi.org/10.1016/s0076-6879\(02\)4846-6](https://doi.org/10.1016/s0076-6879(02)4846-6)
  25. Sindhu RK, Ehdaie A, Farmand F, Dhaliwal KK, Nguyen T, Zhan CD, Roberts CK, Vaziri ND (2005) Expression of catalase and glutathione peroxidase in renal insufficiency. *Biochim Biophys Acta* 1743(1–2):86–92. <https://doi.org/10.1016/j.bbamcr.2004.08.013>
  26. Drozd R, Pilarczyk R, Pilarczyk B, Drozd A, Tomza-Marciniak A, Bombik T, Bąkowska M, Bombik E, Jankowiak D, Wasak A (2015) Activity of selected antioxidant enzymes, selenium content and fatty acid composition in the liver of the brown hare (*Lepus europaeus* L.) in relation to the season of the year. *Biol Trace Elem Res* 168(2):421–428. <https://doi.org/10.1007/s12011-015-0385-5>
  27. Tan AS, Berridge MV (2000) Superoxide produced by activated neutrophils efficiently reduces the tetrazolium salt, WST-1 to produce a soluble formazan: a simple colorimetric assay for measuring respiratory burst activation and for screening anti-inflammatory agents. *J Immunol Methods* 238(1–2):59–68. [https://doi.org/10.1016/s0022-1759\(00\)00156-3](https://doi.org/10.1016/s0022-1759(00)00156-3)
  28. Dawn-Linsley M, Ekinci FJ, Ortiz D, Rogers E, Shea TB (2005) Monitoring thiobarbituric acid-reactive substances (TBARs) as an assay for oxidative damage in neuronal cultures and central nervous system. *J Neurosci Methods* 141(2):219–222. <https://doi.org/10.1016/j.jneumeth.2004.06.010>
  29. Bressler J, Kim KA, Chakraborti T, Goldstein G (1999) Molecular mechanisms of lead neurotoxicity. *Neurochem Res* 24(4):595–600. <https://doi.org/10.1023/a:1022596115897>
  30. Dobrakowski M, Boroń M, Birkner E, Kasperczyk A, Chwalińska E, Lisowska G, Kasperczyk S (2017) The effect of a short-term exposure to lead on the levels of essential metal ions, selected proteins related to them, and oxidative stress parameters in humans. *Oxid Med Cell Longev*. <https://doi.org/10.1155/2017/8763793>
  31. Pedersen S, Lierhagen S (2006) Heavy metal accumulation in arctic hares (*Lepus arcticus*) in Nunavut, Canada. *Sci Total Environ* 368(2–3):951–955. <https://doi.org/10.1016/j.scitotenv.2006.05.014>
  32. Amuno S, Niyogi S, Amuno M, Attitaq J (2016) Heavy metal bioaccumulation and histopathological alterations in wild Arctic hares (*Lepus arcticus*) inhabiting a former lead-zinc mine in the Canadian high Arctic: a preliminary study. *Sci Total Environ* 556:252–263. <https://doi.org/10.1016/j.scitotenv.2016.03.007>
  33. Massányi P, Tataruch F, Slameka J, Toman R, Jurík R (2003) Accumulation of lead, cadmium, and mercury in liver and kidney of the brown hare (*Lepus europaeus*) in relation to the season, age, and sex in the West Slovakian lowland. *J Environ Sci Health Toxic/Hazard Subst Environ Eng* 38(7):1299–1309. <https://doi.org/10.1081/ESE-120021127>
  34. Kolesarova A, Slamecka J, Jurcik R, Tataruch F, Lukac N, Kovacic J, Capcarova M, Valent M, Massanyi P (2008) Environmental levels of cadmium, lead and mercury in brown hares and their relation to blood metabolic parameters. *J Environ Sci Health Toxic/Hazard Subst Environ Eng* 43(6):646–650. <https://doi.org/10.1080/10934520801893741>
  35. Patrick L (2006) Lead toxicity part II: the role of free radical damage and the use of antioxidants in the pathology and treatment of lead toxicity. *Altern Med Rev* 11(2):114–127
  36. Mates JM (2000) Effects of antioxidant enzymes in the molecular control of reactive oxygen species toxicology. *Toxicology* 153(1–3):83–104. [https://doi.org/10.1016/s0300-483x\(00\)00306-1](https://doi.org/10.1016/s0300-483x(00)00306-1)
  37. Gurer H, Ercal N (2000) Can antioxidants be beneficial in the treatment of lead poisoning? *Free Radic Biol Med* 29(10):927–945. [https://doi.org/10.1016/s0891-5849\(00\)00413-5](https://doi.org/10.1016/s0891-5849(00)00413-5)
  38. Sandhir R, Julka D, Gill KD (1994) Lipoperoxidative damage on lead exposure in rat brain and its implications on membrane bound enzymes. *Pharmacol Toxicol* 74(2):66–71. <https://doi.org/10.1111/j.1600-0773.1994.tb01077.x>
  39. Limón-Pacheco J, Gonsebatt ME (2009) The role of antioxidants and antioxidant-related enzymes in protective responses to environmentally induced oxidative stress. *Mutat Res Genet Toxicol*

- Environ Mutagen 674(1–2):137–147. <https://doi.org/10.1016/j.mrgentox.2008.09.015>
40. Obolenskayaa MY, Teplyuka NM, Divi RL, Poirier MC, Filimonovac NB, Zadrozna M, Pasanene MJ (2010) Human placental glutathione S-transferase activity and polycyclic aromatic hydrocarbon DNA adducts as biomarkers for environmental oxidative stress in placentas from pregnant women living in radioactivity- and chemically-polluted regions. *Toxicol Lett* 196(2):80–86. <https://doi.org/10.1016/j.toxlet.2010.03.1115>
  41. Gürer H, Ozgünes H, Neal R, Spitz DR, Erçal N (1998) Antioxidant effects of N-acetylcysteine and succimer in red blood cells from lead-exposed rats. *Toxicology* 128(3):181–189. [https://doi.org/10.1016/s0300-483x\(98\)00074-2](https://doi.org/10.1016/s0300-483x(98)00074-2)
  42. Mohammad IK, Mahdi AA, Raviraja A, Najmul I, Iqbal A, Thuppil V (2008) Oxidative stress in painters exposed to low lead levels. *Arh Hig Rada Toksikol* 59(3):161–169. <https://doi.org/10.2478/10004-1254-59-2008-1883>

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